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ANALYSIS OF EFFECT OF *WOLBACHIA* SURFACE PROTEIN (WSP) ON APOPTOSIS AND MITOCHONDRIAL MEMBRANE POTENTIAL IN ETHANOL-INDUCED TOXICITY OF HepG2 CELL LINE

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ABSTRACT

Aim: *To study the effect of Wolbachia Surface Protein (WSP) on Apoptosis and Mitochondrial Membrane Potential in Ethanol-exposed HepG2 cells.* ***Materials and Methods:*** *Toxicity was induced in HepG2 cells by incubating HepG2 cells with Ethanol. Effect of Wolbachia Surface Protein (WSP) on Apoptosis and Mitochondrial Membrane Potential were determined by flow cytometry using Annexin V-FITC, PI and JC-1 stains respectively.* ***Results and Discussion:*** *Study of Annexin V-FITC and PI dual staining demonstrated that incubation of HepG2 cells with Ethanol induced apoptosis and the percentage of this Ethanol-induced apoptosis in the cells were decreased in the presence of Wolbachia Surface Protein (WSP). Study of JC-1 staining demonstrated that incubation of HepG2 cells with Ethanol induced depolarization of mitochondrial membrane and the percentage of this Ethanol-induced depolarization of mitochondrial membrane in the cells were decreased in the presence of Wolbachia Surface Protein (WSP). Thus, these findings indicate that the treatment with Wolbachia Surface Protein (WSP) inhibited Apoptosis and Mitochondrial Membrane depolarization in Ethanol-induced toxicity of HepG2 cells. Taken together, our results reveal that Wolbachia Surface Protein (WSP) shows antiapoptotic activity and inhibition of mitochondrial membrane damage.* ***Conclusion:*** *We can conclude that Wolbachia Surface Protein (WSP) demonstrates cytoprotective effect against Ethanol-induced Apoptosis and Mitochondrial membrane depolarization in HepG2 cells by reducing the Apoptosis and depolarization of mitochondrial membrane. This will be of interest for the treatment of Ethanol-related liver diseases. Thus, Wolbachia Surface Protein (WSP) with potential protective activity might be used as therapeutic drug to treat Ethanol-related liver diseases.*

KEY WORDS

Apoptosis, Flow cytometry, HepG2 cells, Mitochondrial Membrane Potential (MMP) (Δψm), Therapeutic drug, Wolbachia Surface Protein (WSP).

INTRODUCTION

Wolbachia surface protein (WSP) is the most profusely expressed protein of an endosymbiotic Gram-negative bacteria *Wolbachia*. Previous studies showed that rWSP causes apoptosis of monocytes and not lymphocytes [1]. Another study by Bazzocchi *et al.,* has revealed that WSP, a surface protein of the endosymbiotic bacteria of filarial nematodes have anti-apoptotic activity by slowing down apoptosis in human polymorphonuclear cells PMNs [2]. Motivating observation of the earlier reports inspired us to study the effect of WSP on Ethanol induced toxicity in HepG2 cells. Even though there is information present on the effect of WSP on different human cells but the information on the effect of WSP (obtained from *Wolbachia* of *Exorista sorbillans*) on Ethanol induced toxicity with respect to flow cytometric determination of Apoptosis and Mitochondrial

Membrane Potential in HepG2 cells has not been known so far.

Apoptosis is a form of programmed cell death (PCD) [3]. Apoptosis is an important mechanism which occurs in response to both normal physiological processes and in response to a variety of stimuli [4]. Mitochondria are considered to play an important role in apoptosis [5]. Flow cytometric methods like determination of Externalization of Phosphatidylserine, Dissipation of Mitochondrial Transmembrane Potential, Caspase activation, DNA strand breaks (TUNEL Assay) etc., can be used to detect Apoptosis [6]. Thus, in this article we have determined and discussed the effects of WSP on Apoptosis and Mitochondrial Membrane Potential in Ethanol-induced toxicity of HepG2 cells by examining the Phosphatidyl Serine exposure and mitochondrial membrane depolarization using flow cytometer.

As WSP will be derived from *Wolbachia*, the effect of WSP might vary relying on the nature of *Wolbachia* being used which may depend on the source of *Wolbachia* and the effect of WSP might also vary depending on the nature of the cells in which the WSP comes in contact. So here in this study *Wolbachia* surface protein (WSP), a protein of *Wolbachia* obtained from *Exorista sorbillans* was used. HepG2 cells were chosen and used for studying the effect of WSP on Ethanol induced toxicity in HepG2 cells as HepG2 cell lines are used for the examination of cytotoxic and cytoprotective substances [7]. Ethanol was used for inducing toxicity in HepG2 cells as Ethanol is known to induce toxicity in HepG2 cells [8].

Keeping all the above points in view, here in this study we studied the effect of WSP (obtained from *Wolbachia* of *Exorista sorbillans*) on Apoptosis and Mitochondrial Membrane Potential (MMP) in Ethanol induced toxicity of HepG2 cells by flow cytometry.

In this study, we examined the effect of *Wolbachia* Surface Protein (WSP) on Apoptosis and Mitochondrial Membrane Potential (MMP) ($\Delta\Psi m$) in HepG2 cells in response to Ethanol induced cytotoxicity. To examine the effect of *Wolbachia* Surface Protein (WSP), toxicity was induced in HepG2 cells by incubating HepG2 cells with Ethanol. Apoptosis and Mitochondrial Membrane Potential were determined by flow cytometry. Apoptosis was assessed by Annexin V-Fluorescein isothiocyanate (FITC) and Propidium Iodide (PI) staining. Annexin V-Fluorescein isothiocyanate (FITC) stains externalized Phosphatidylserine (PS) on the plasma membrane, which is the hallmark of apoptosis [9, 10]. Propidium Iodide (PI) was used to differentiate dead and apoptotic cells [11] on the basis of uptake of Propidium Iodide by the dead and damaged cells [12]. Study of Annexin V-FITC and PI dual staining demonstrated that incubation of HepG2 cells with Ethanol induced apoptosis and the percentage of this Ethanol-induced apoptosis in the cells were decreased in the presence of *Wolbachia* Surface Protein (WSP). The Mitochondrial Membrane Potential was assessed by the JC-1 staining. JC-1 emits either red or green fluorescence, relying on the mitochondrial membrane potential, red signal representing polarized mitochondria, while the green signal representing depolarized mitochondria [13]. Study of JC-1 staining demonstrated that incubation of HepG2 cells with Ethanol induced depolarization of mitochondrial membrane and the percentage of this Ethanol-induced depolarization of mitochondrial membrane in the cells were decreased in the presence of *Wolbachia* Surface Protein (WSP). The cytoprotective effects of *Wolbachia* Surface Protein (WSP) were comparable with that of Silymarin, a known hepatoprotective agent [14]. Thus, these findings indicated that treatment with *Wolbachia* Surface Protein (WSP) protected against Apoptosis and Mitochondrial membrane depolarization by reducing the Apoptosis and depolarization of mitochondrial membrane. Thus, our data indicate that *Wolbachia* Surface Protein (WSP) has the ability to protect against Apoptosis and Mitochondrial membrane depolarization in EtOH-exposed HepG2 cells. These data suggest that *Wolbachia* Surface Protein (WSP) might serve as a useful potential therapeutic drug in the treatment of Ethanol-related liver diseases.

MATERIALS AND METHODS

Chemicals and Materials

FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) (Catalog No. 556547) and BD™ MitoScreen Flow Cytometry Mitochondrial Membrane Potential Detection Kit [Becton, Dickinson and Company BD Biosciences (San Jose, CA, USA)] (Catalog No. 551302) was purchased from Becton, Dickinson and Company BD Biosciences (San Jose, CA, USA).

Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), L-glutamine, Penicillin, Streptomycin, Trypsin-EDTA and Phosphate Buffered

Saline (PBS) were purchased from Sigma Chemical Company, St. Louis, MO, USA. Cell culture flasks and culture plates were purchased from Fisher Scientific (Pittsburgh, PA).

Unless otherwise stated all other chemicals, reagents and materials used in this study were purchased from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals used in the present study were all of the analytical grades.

Production of recombinant *Wolbachia* surface protein (rWSP) by cloning, expression and purification

Production of recombinant *Wolbachia* Surface Protein (rWSP) was essential for studying their effect on Apoptosis and Mitochondrial membrane potential in Ethanol induced toxicity of HepG2 cells.

The *Wolbachia* surface protein (WSP) of the *Wolbachia* from *Exorista sorbillans* was produced in recombinant form by cloning, expression and purification as described below.

The gene encoding for the *Wolbachia* Surface Protein (WSP), obtained from genomic DNA extracted from *Exorista sorbillans*, was amplified by polymerase chain reaction (PCR) using the primers. The primer sequence of forward primer used was 5' – CGA ATT CAT ATG GAT CCT GTT GGT CCA ATA AGT G – 3' and the primer sequence of reverse primer used was 5' – GCC TCG AGT CTA GAC CTA GAA ATT AAA CGC TAC – 3'. The forward primer and the reverse primer was tagged with the NdeI and the XhoI restriction enzyme sites respectively. Therefore, the amplified PCR product (WSP gene) obtained was cloned into the pET 19b Expression vector. The pET 19b-WSP plasmid was transferred into the competent cells of *Escherichia coli* DH5-α. The transferred clones were examined for the presence of WSP gene by means of Electrophoretic mobility analysis. Retardation in electrophoretic mobility was observed in the clones having the WSP gene when compared to that of the clones without WSP gene (data not shown). Retardation seen in the electrophoretic mobility of the clones proved the presence of WSP gene. The presence of the WSP gene in the retarded clones were further examined and proved by PCR examination based on the amplification of the WSP gene using primers. Amplification of the WSP gene using primers proved the presence of WSP gene in the retarded clones (data not shown). Clones proved for the presence of WSP gene was transferred into *Escherichia coli* BL21 (DE3) and the expression of the His-Tagged recombinant WSP (His-Tagged rWSP) was induced by 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). After induction, the cells were incubated at 37°C for 4 h, then 1.5 ml culture was taken and centrifuged at 6000 rpm for 5 minutes. The cell pellet obtained was suspended in 50 µl of 1X TE and 5 µl of Cell Lysis buffer (containing 40 mg/ml Lysozyme supplemented with 800 U/ml DNase and 24 U/ml RNase) was added. This was incubated at 37°C for 30-60 minutes and centrifuged at 8000 rpm for 5 minutes. To the pellet (insoluble protein), 6 M Urea was added. This induced and lysed culture, that is clones were examined for the expression by loading on 12% Sodium Dodecyl Sulphate Polyacryl Amide Gel Electrophoresis (SDS PAGE) gel and performing Sodium Dodecyl Sulphate Polyacryl Amide Gel Electrophoresis at 100 V for 4 h proceeded by staining and destaining (data not shown). Clones which had very good expression was used for further processing of Purification and Dialysis.

The recombinant His-tagged WSP expressed was purified by immobilized metal affinity chromatography (IMAC) using Nickel affinity absorption chromatography that is nickel-nitrilotriacetic acid (Ni-NTA) column. The cell pellet having the recombinant protein was solubilized by sonication with 6 M Urea with sonication conditions of 30% ampt and 40°C until the optical density of cells reached 1/10th at 600 nm Absorbance. The soluble proteins were separated by centrifugation at 15,000 rpm for 20 minutes at 4°C and the supernatant was used for purification of the recombinant protein. Supernatant containing the recombinant protein was purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity column. Ni-NTA column was equilibrated using Cell lysis buffer with 6 M Urea. The supernatant (soluble protein) was loaded onto the pre-equilibrated column. Unbound protein was washed with the wash buffer containing 10 mM Imidazole and the bound protein was eluted using a step elution of Imidazole (an imidazole gradient) (100 mM-300 mM Imidazole) in the Cell lysis buffer with 6 M Urea. The eluted protein is the purified protein. The purified protein was dialyzed against 1X PBS buffer with 4 M Urea for 2 h using dialysis membrane at 4°C. Buffer was changed with fresh 1X PBS with 2 M Urea and again dialyzed for 2 h at 4°C. Final dialysis was done with buffer 1X PBS for 2 h at 4°C. After dialysis, the dialyzed protein was examined and proved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) (data not shown). The dialyzed protein (*Wolbachia*

Surface Protein) was transferred into a bottle and stored at 4°C until further usage.

The *Wolbachia* Surface Protein (WSP) was thus produced by cloning, expression and purification (data not shown).

The recombinant *Wolbachia* Surface Protein (WSP) thus produced was further used to study their effect on Apoptosis and Mitochondrial membrane potential in Ethanol induced toxicity of HepG2 cells.

HepG2 cell line and culture conditions

HepG2 cell line was used in this study. HepG2 cells were taken from the National Centre for Cell Science (NCCS) Pune, India. HepG2 cells were cultured and kept in cell culture flasks having Dulbecco's Modified Eagle's Medium (DMEM) added with 10% Fetal Bovine Serum (FBS), Penicillin (100 U/ml), Streptomycin (100 µg/ ml) and 2 mM L-glutamine in a 5% CO_2 incubator at 37°C.

Cell Treatments

Cells were grown until they became confluent. On getting 75–80% confluence, the cells were treated with 56.57 mM Ethanol (EtOH) alone, EtOH + 150 µg/ml *Wolbachia* Surface Protein (WSP) and EtOH + 250 µM Silymarin (SIL) for 24 h. Primary experiments were carried out to fix the time of exposure and dosage of EtOH, WSP and Silymarin on the basis of cell viability analysis by MTT assay (data not given). After the end of incubation, cells were used for the various assays elucidated below.

Determination of Apoptosis by Flow cytometry using Annexin V-Fluorescein isothiocyanate (FITC) staining

Apoptosis was determined by FACSCalibur Flow cytometer (Becton Dickinson (BD) Biosciences, San Jose, CA, USA) using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) following the manufacturer's instructions. Apoptosis was analyzed by double staining with Annexin V–fluorescein isothiocyanate (FITC) and Propidium iodide (PI).

Fluorescein isothiocyanate (FITC) conjugated Annexin V (a 35-36 kDa, Calcium-reliant phospholipid-binding protein with great affinity for Phosphatidylserine) detects apoptosis by staining the externalized Phosphatidylserine on the plasma membrane, which is the hallmark of apoptosis [9, 10]. Propidium iodide (PI) was used to differentiate dead and apoptotic cells [11] on the basis of uptake of Propidium iodide by the dead and damaged cells [12]. Propidium iodide (PI) the red-fluorescent, nucleic acid binding dye stains necrotic cells and they are impenetrable to live and apoptotic cells. Apoptotic cells stained with Annexin V exhibit green fluorescence, dead cells exhibit red and green fluorescence and viable cells exhibit slight or no fluorescence [15].

To assess Apoptosis, HepG2 cells were cultured at a density of 3 × 10^5 cells/2 ml in a 6-well plate and incubated in a CO_2 incubator at 37°C for 24 h. The spent medium was aspirated and washed with 1 ml 1X PBS. Then the cells were incubated for 24 h in the presence and absence of 56.57 mM Ethanol (EtOH) discretely and were then treated for 24 h in the presence of 150 µg/ml *Wolbachia* Surface Protein (WSP) and 250 µM Silymarin (SIL) discretely. After WSP and Silymarin treatment, the medium was removed from all the wells, transferred to the tubes and washed with 500 µl PBS (the PBS was kept inside the same tubes). Cells kept as Untreated (UT) were used as control. The PBS was discarded, added 180 µl of Trypsin-EDTA solution and incubated for 3-4 minutes at 37°C. The culture medium was poured back into their own wells, the cells were harvested and poured into the tubes. The tubes having the cells were subjected to centrifugation for 5 minutes at 300 g at 25°C. The supernatant was discarded carefully and the pellet was washed with PBS twice. The PBS was totally discarded. At a concentration of 1X10^6 cells/ml, cells were resuspended in 1X Binding Buffer. To a 5 ml culture tube, 100 µl of the solution (1X10^5 cells) was transferred. 5 µl of Annexin V-FITC and 5 µl of Propidium iodide was added. The cells were gently vortexed and were then incubated for 15 minutes at room temperature (25°C) in the dark. 400 µl of 1X Binding Buffer was added to each tube. Cells were analyzed by FACSCalibur flow cytometer within 1 h.

Flow cytometry studies and data analysis of Apoptosis

Flow cytometry determination of Apoptosis in HepG2 cells were done using a FACSCalibur flow cytometer (Becton Dickinson (BD) Biosciences, San Jose, CA, USA). Data were acquired and analyzed using FACSCalibur flow cytometer equipped with a 15 mW, 488 nm air-cooled argon-ion laser and CellQuest Pro software (Becton Dickinson BD Biosciences, San Jose, CA, USA). To avoid nonspecific fluorescence from dead cells, live cells were gated using forward and side scatter. Cells were examined for each sample.

Apoptosis in HepG2 cells were analyzed by staining using Annexin V-Fluorescein isothiocyanate (FITC) and Propidium iodide (PI). The green fluorescence (FITC) and red fluorescence (PI) were detected by filtration

through a 530 nm bandpass filter in the FL-1 (FITC) channel for cells labeled with Annexin V-FITC and a 585 nm bandpass filter in the FL-2 (PI) channel for cells labeled with PI respectively. Then an FL-1/FL-2 dot plot was generated. Histograms were analyzed for Annexin V and Propidium iodide labelled cells. Histograms show log scale of fluorescence intensity on the X-axis and cell count on the Y-axis. Quadrant analysis of coordinated dot plots were done. The Lower Left (LL) quadrant depicts the live cells, which are negative for Annexin V-FITC and PI (Annexin V-FITC-, PI-). The Lower Right (LR) quadrant depicts the early apoptotic cells, which are positive for Annexin V-FITC and negative for PI (Annexin V-FITC+, PI-). The Upper Left (UL) quadrant depicts the necrotic cells, which are negative for Annexin V-FITC and positive for PI (Annexin V-FITC-, PI+). The Upper Right (UR) quadrant depicts late apoptotic cells, which are positive for both Annexin V-FITC and PI (Annexin V-FITC+, PI+) [16].

Determination of Mitochondrial Membrane Potential (ΔΨm) (MMP) by Flow cytometry using JC-1 staining

Mitochondrial membrane potential (Δψm) (MMP) was assessed by FACSCalibur Flow cytometer (Becton Dickinson (BD) Biosciences, San Jose, CA, USA) using the BD™ MitoScreen Flow Cytometry Mitochondrial Membrane Potential Detection Kit [Becton, Dickinson and Company BD Biosciences (San Jose, CA, USA)] following the manufacturer's protocol. MMP (ΔΨm) in HepG2 cells were analyzed using 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1).

JC-1 detection of Mitochondrial membrane potential is based on the fact that JC-1 emits either red or green fluorescence, relying on the mitochondrial membrane potential, red signal representing polarized mitochondria, while the green signal representing depolarized mitochondria [13]. The lipophilic, fluorescent cationic dye, JC-1 exists as monomers at low mitochondrial membrane potential and form aggregates at high mitochondrial membrane potential [17, 18, 19]. In hale cells having high level of mitochondrial membrane potential, JC-1 instinctively creates complexes in mitochondria called as J-aggregates with extreme red fluorescence. In case of apoptotic or unhealthy cells having low mitochondrial membrane potential, JC-1 cannot gather in the mitochondria, will be in the cytoplasm in the monomeric form and exhibits only green fluorescence [20].

To assess Mitochondrial membrane potential, HepG2 cells were cultured to an optimal density (not exceeding 1×10^6 cells per ml) and were incubated for 24 h in the presence and absence of 56.57 mM Ethanol (EtOH) discretely and were then treated for 24 h in the presence of 150 µg/ml *Wolbachia* Surface Protein (WSP) and 250 µM Silymarin (SIL) discretely. 1 ml of each cell suspension was transferred into a sterile 15 ml polystyrene centrifuge tube at the end of the treatment period. Cells kept as Untreated (UT) were used as control. Cells were centrifuged at 400 g for 5 minutes at room temperature. The supernatant was carefully removed and discarded. 0.5 ml of freshly prepared JC-1 working solution (working solution of 1X JC-1 was prepared by diluting the JC-1 Stock Solution 1:100 with prewarmed 1X assay buffer. That is, 125 µl JC-1 stock was added to 12.375 ml of prewarmed assay buffer) was added to each pellet. Cells in the JC-1 working solution was resuspended gently. Cell-to-cell clumping, if any was disrupted by vortexing or using a pipette. The cells in JC-1 working solution were incubated for 10 – 15 minutes at 37°C in a CO_2 incubator. After incubation, cells were washed twice by following wash steps (1^{st} wash and 2^{nd} wash) at room temperature. 1^{st} wash: adding 2 ml of 1X assay buffer to each tube and resuspending the cells gently. Cell-to-cell clumping, if any was disrupted by vortexing or using a pipette. The cells were centrifuged at 400 g for 5 minutes. The supernatant was removed carefully and discarded. 2^{nd} wash: 1 ml of 1X assay buffer was added to each tube and the cells were resuspended gently. Cell-to-cell clumping, if any was disrupted by vortexing or using a pipette. The cells were centrifuged at 400 g for 5 minutes. Each cell pellet was gently resuspended in 0.5 ml of 1X assay buffer. Cell-to-cell clumping, if any was disrupted by vortexing or using a pipette. Cells were analyzed by FACSCalibur flow cytometer.

Flow cytometry studies and data analysis of Mitochondrial Membrane Potential (ΔΨm) (MMP)

Flow cytometry determination of mitochondrial membrane potential in HepG2 cells were done using a FACSCalibur flow cytometer (Becton Dickinson (BD) Biosciences, San Jose, CA, USA). Data acquisition was performed in a FACSCalibur flow cytometer equipped with a 15 mW, 488 nm air-cooled argon-ion laser. Data from the experiments were analyzed using CellQuest

Pro software (Becton Dickinson (BD) Biosciences, San Jose, CA, USA). To exclude debris, samples were gated based on light scattering properties in the side scattering (SSC) and forward scattering (FSC) modes and cells per sample within the gate (R1) were collected.

Mitochondrial membrane potential was analyzed by staining using the fluorescent probe JC-1. JC-1 was excited at 488 nm and the JC-1 monomer fluorescence signal (green fluorescence) was analyzed at 527 nm on the FL1 detector and the JC-1 aggregate fluorescence signal (red fluorescence) was analyzed at 590 nm on the FL2 detector on a FACSCalibur flow cytometer. The red and green fluorescence were measured simultaneously by FACSCalibur flow cytometer. Cells with normal polarized mitochondrial membranes emit green-orange fluorescence and are found in the upper right quadrant; those with depolarized mitochondrial membranes emit only green fluorescence and are found in the lower right quadrant of FACS Quadrant plot [21]. Mitochondrial Membrane Depolarization is associated with a large shift in JC-1 fluorescence emission from red to green [22].

Statistical analysis

All Statistical analysis were performed using the statistical software GraphPad Prism 6.0 Software (GraphPad Software, Inc., San Diego, CA, USA). Data were expressed as the mean ± Standard Deviation (SD) of three independent experiments performed. Data were statistically analyzed using Analysis of Variance (ANOVA) followed by Tukey's test. p-values less than 0.05 ($p<0.05$) were considered to be statistically significant.

RESULTS AND DISCUSSION

Flow cytometric analysis of effect of *Wolbachia* Surface Protein (WSP) on Apoptosis in HepG2 cells exposed to Ethanol

We examined the effect of *Wolbachia* Surface Protein (WSP) on Apoptosis in HepG2 cells by incubating with and without 56.57 mM Ethanol (EtOH) discretely for 24 h followed by treatment with 150 µg/ml *Wolbachia* Surface Protein (WSP) and 250 µM Silymarin (SIL) discretely for 24 h. Then the Apoptosis was analyzed by Annexin V-FITC and PI staining and was quantified using FACSCalibur flow cytometer (Becton Dickinson (BD) Biosciences, San Jose, CA, USA).

Cells were acquired for analysis by CellQuest Pro software (Becton Dickinson (BD) Biosciences, San Jose, CA, USA) and quantification of apoptotic cells, as the percentage of the total cell population was performed. We analyzed the live, apoptotic and dead cells using FACSCalibur flow cytometer by staining cells with Annexin V-FITC and PI. By detection of Annexin V-FITC and PI fluorescence, four different populations of cells were easily identified by Quadrant analysis. The percentages of Live cells stained negative for both Annexin V-FITC and PI (Annexin V-FITC −/PI−), cells in the early stages of apoptosis stained positive for Annexin V-FITC and negative for PI (Annexin V-FITC+/PI-), late apoptotic cells stained positive for both Annexin V-FITC and PI (Annexin V-FITC+/ PI+) and necrotic cells stained negative for Annexin V-FITC and positive for PI (Annexin V-FITC−/PI+) cells.

We observed that Apoptosis level was little in Untreated (UT) HepG2 cells. Apoptosis was observed significantly in HepG2 cells exposed to Ethanol (EtOH) for 24 h. *Wolbachia* Surface Protein (WSP) treatment for 24 h reduced Apoptosis in Ethanol exposed HepG2 cells and Silymarin (SIL) treatment for 24 h also reduced Apoptosis in Ethanol exposed HepG2 cells as determined by flow cytometer using Annexin V-FITC and PI stain (Figure 1, 2, 3, 4) (Table 1) (Figure 5).

We found that the treatment of Ethanol exposed HepG2 cells with the *Wolbachia* Surface Protein (WSP) decreased EtOH-induced Apoptosis. Therefore, in summary, the present study suggests that *Wolbachia* Surface Protein (WSP) protected HepG2 cells against Apoptosis induced by Ethanol. These data suggest that *Wolbachia* Surface Protein (WSP) might act as cytoprotective agent.

Literature study showed that *Wolbachia* Surface Protein (WSP) hinders apoptosis in human Polymorphonulear cells (PMNs) [2]. However, there was no experimental evidence regarding WSP's (of *Wolbachia* of Uzifly) protection against Ethanol induced apoptosis in HepG2 cells with respect to flow cytometric determination. These findings clearly showed that WSP could protect the HepG2 cells from Apoptosis and cytotoxic effects of Ethanol. WSP has a similar effect as that of the known hepatoprotective agent, Silymarin [14]. This study suggests that *Wolbachia* Surface Protein (WSP) might be used as an effective therapeutic drug to treat Ethanol-related liver diseases.

Table 1: Data for percentage of Apoptosis in HepG2 cells

Groups	Percentage of Apoptosis
Untreated (UT)	0.1933 ± 0.01528
EtOH	75.29 ± 2.031
EtOH-WSP	41.17 ± 1.400
EtOH-SIL	22.99 ± 1.649

Values expressed are the mean ± Standard Deviation (SD) of three independent experiments.

Table 2: Data for percentage of depolarization of mitochondrial membrane in HepG2 cells

Groups	Percentage of depolarization of Mitochondrial membrane
Untreated (UT)	0.0033 ± 0.0033
EtOH	82.83 ± 2.003
EtOH-WSP	54.77 ± 2.012
EtOH-SIL	43.57 ± 2.519

Values expressed are the mean ± Standard Deviation (SD) of three independent experiments.

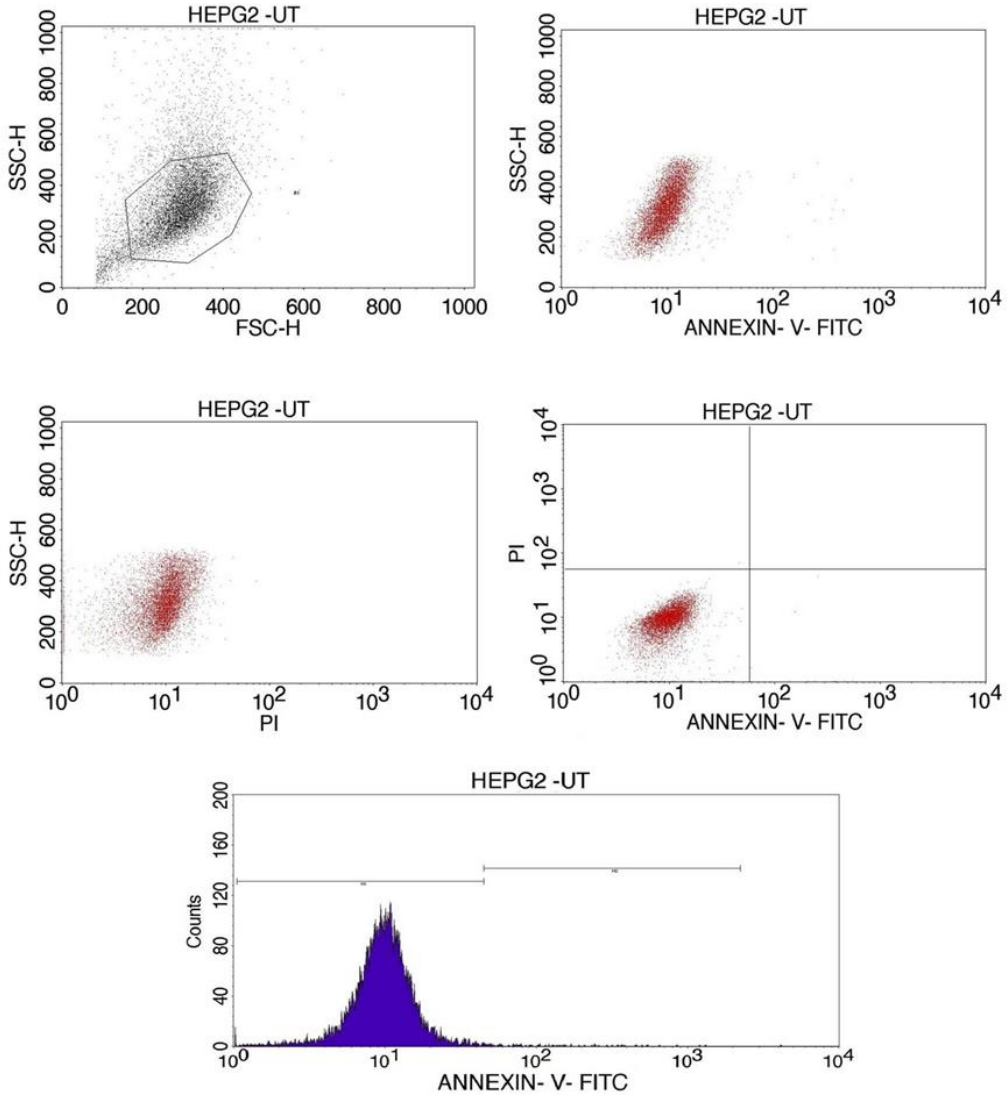

Figure 1: Representative profile of Flow cytometric examination outcome of Apoptosis in Untreated (UT) HepG2 cells

Flow cytometric examination outcome shown is the representative data of 3 individual experiments.

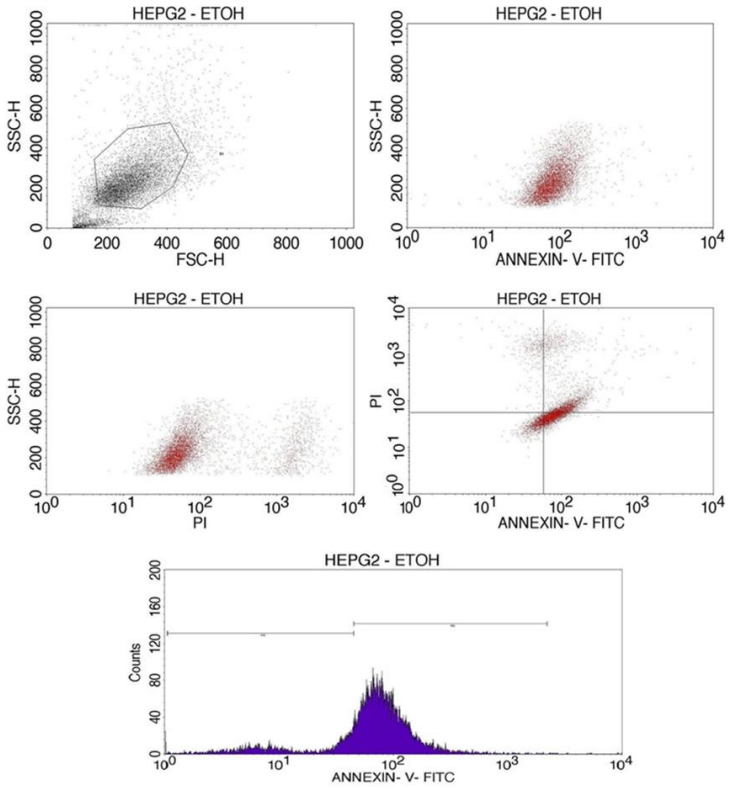

Figure 2: Representative profile of Flow cytometric examination outcome of Apoptosis in HepG2 cells exposed to Ethanol (EtOH) for 24 h

Flow cytometric examination outcome shown is the representative data of 3 individual experiments.

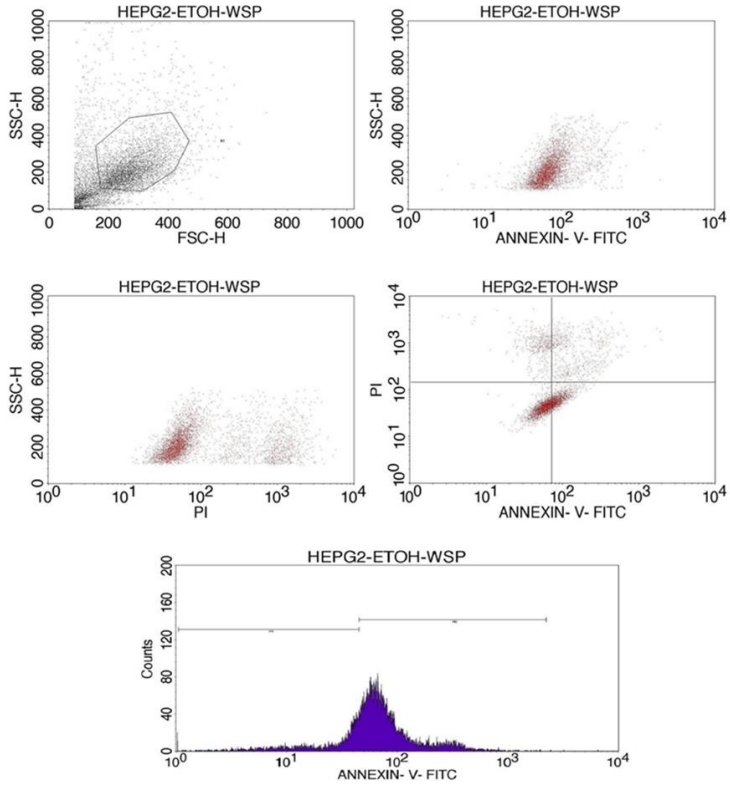

Figure 3: Representative profile of Flow cytometric examination outcome of Apoptosis in the Ethanol exposed HepG2 cells treated with *Wolbachia* Surface Protein (WSP) for 24 h

Flow cytometric examination outcome shown is the representative data of 3 individual experiments.

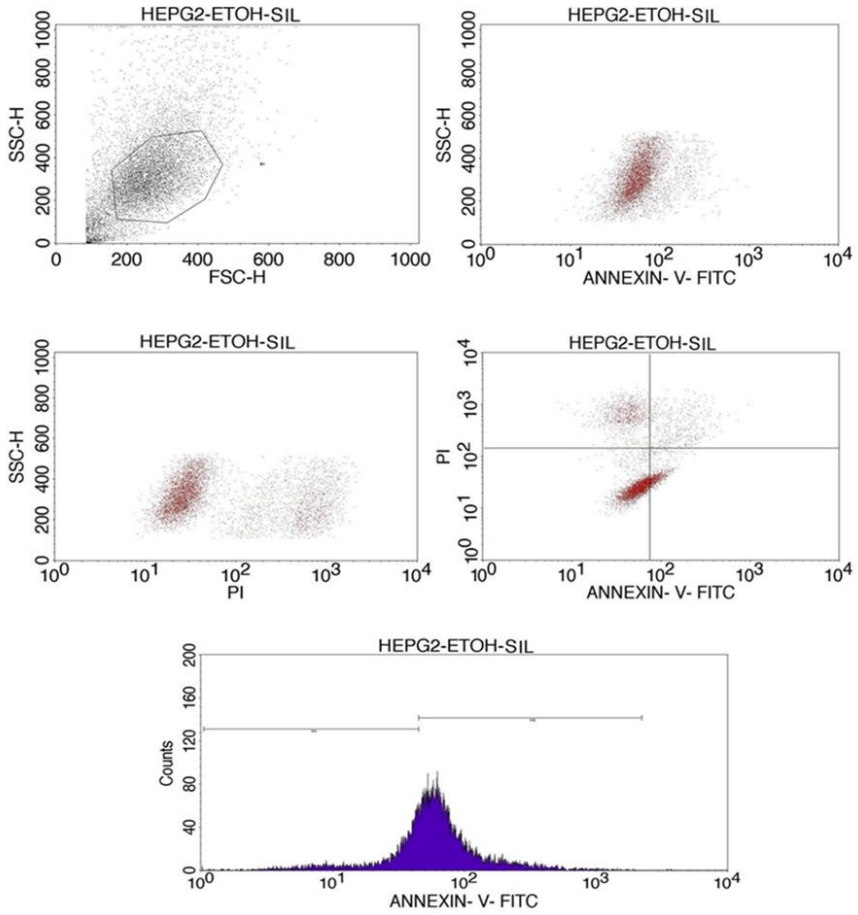

Figure 4: Representative profile of Flow cytometric examination outcome of Apoptosis in the Ethanol exposed HepG2 cells treated with Silymarin (SIL) for 24 h

Flow cytometric examination outcome shown is the representative data of 3 individual experiments.

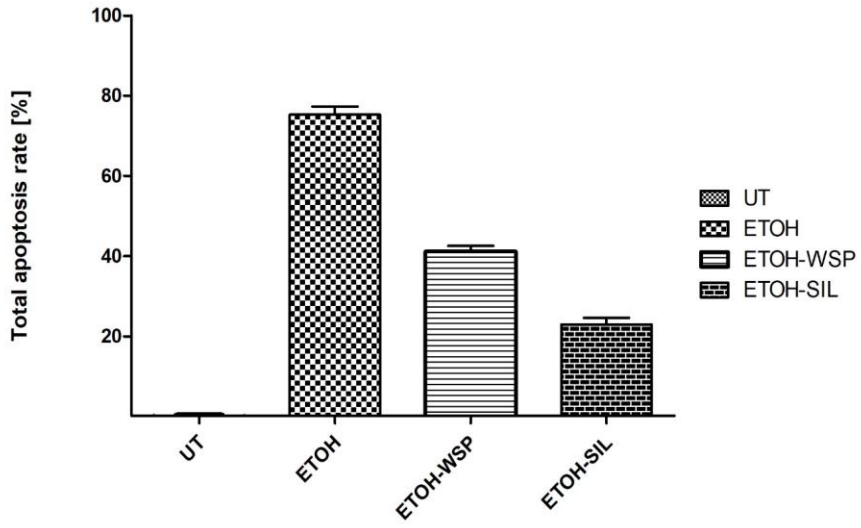

Figure 5: Graphical representation of data for percentage of Apoptosis in HepG2 cells

Data plotted in the bar graph are the mean ± SD of three individual experiments (n=3). Significance: EtOH was found to be highly significant while compared to UT with a p-value of p< 0.0001. EtOH-WSP and EtOH-SIL were found to be highly significant while compared to EtOH with the p-value of p< 0.0001.

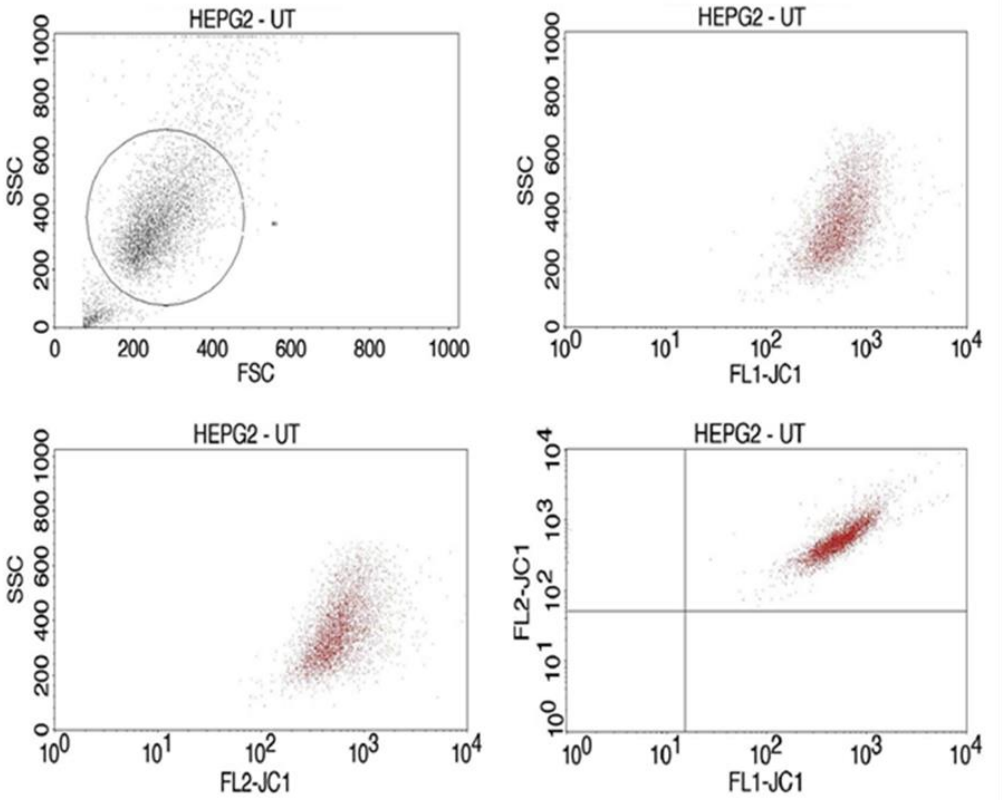

Figure 6: Representative profile of Flow cytometric examination outcome of Mitochondrial Membrane Potential (MMP) in Untreated (UT) HepG2 cells

Flow cytometric examination outcome shown is the representative data of 3 individual experiments.

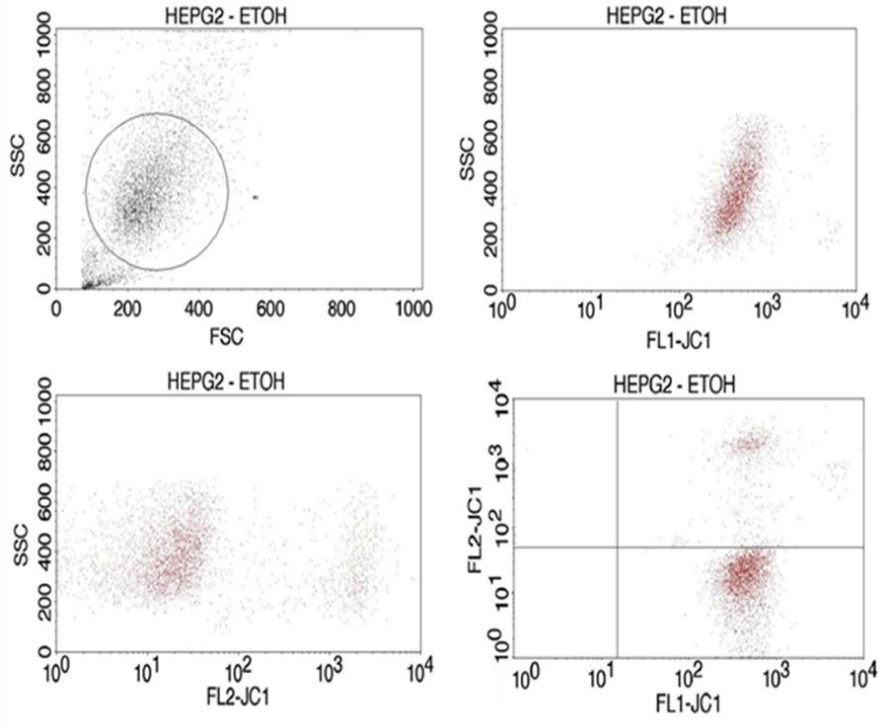

Figure 7: Representative profile of Flow cytometric examination outcome of Mitochondrial Membrane Potential (MMP) in HepG2 cells exposed to Ethanol (EtOH) for 24 h

Flow cytometric examination outcome shown is the representative data of 3 individual experiments.

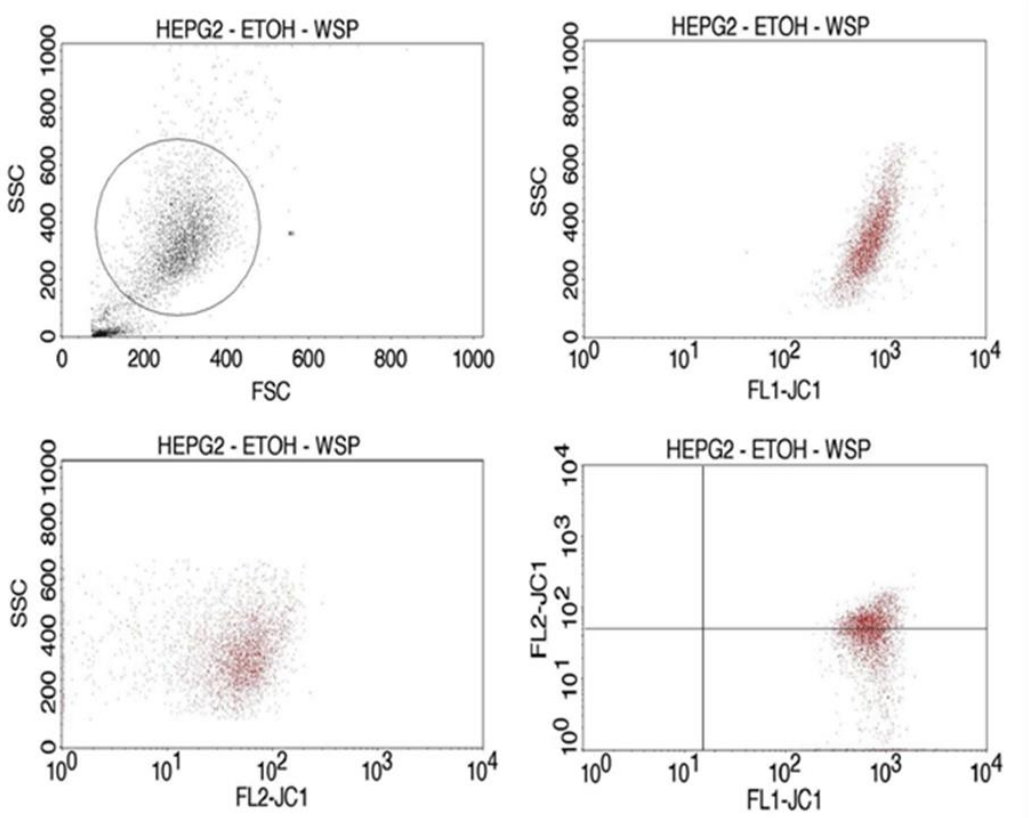

Figure 8: Representative profile of Flow cytometric examination outcome of Mitochondrial Membrane Potential (MMP) in the Ethanol exposed HepG2 cells treated with *Wolbachia* Surface Protein (WSP) for 24 h

Flow cytometric examination outcome shown is the representative data of 3 individual experiments.

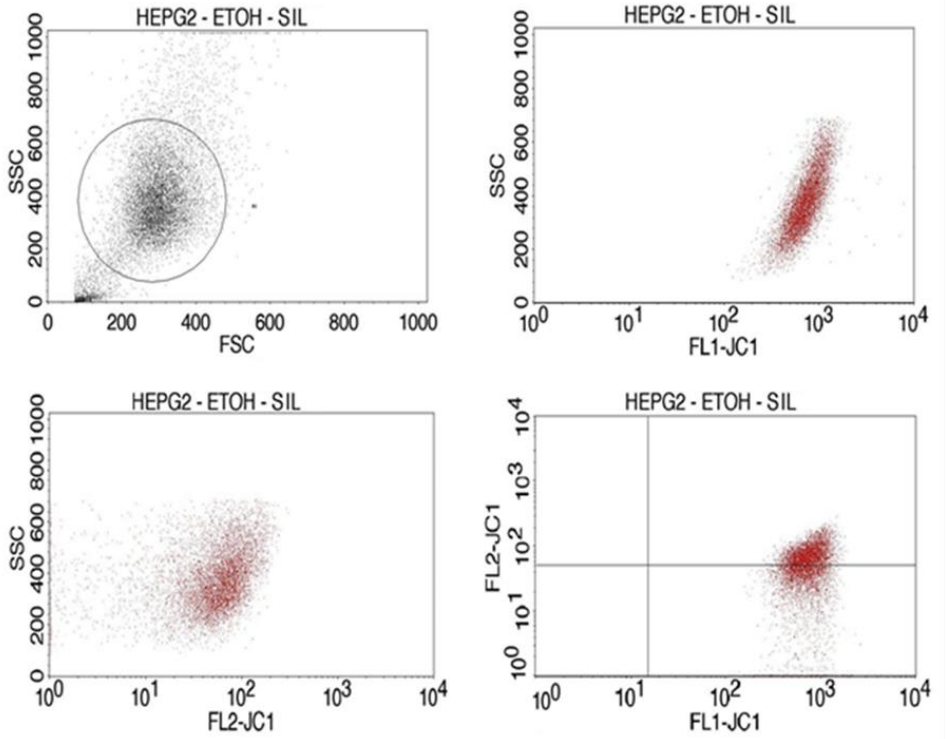

Figure 9: Representative profile of Flow cytometric examination outcome of Mitochondrial Membrane Potential (MMP) in the Ethanol exposed HepG2 cells treated with Silymarin (SIL) for 24 h

Flow cytometric examination outcome shown is the representative data of 3 individual experiments.

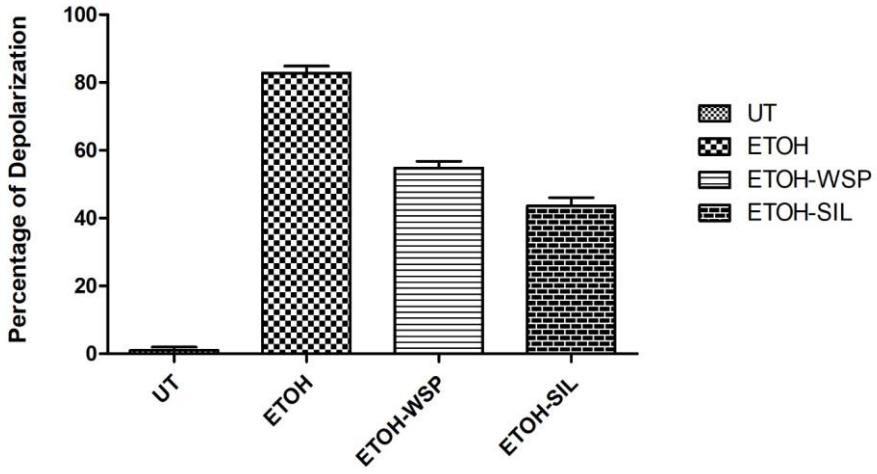

Figure 10: Graphical representation of data for percentage of depolarization of mitochondrial membrane in HepG2 cells

Data plotted in the bar graph are the mean ± SD of three individual experiments (n=3). Significance: EtOH was found to be highly significant while compared to UT with a p-value of p< 0.0001. EtOH-WSP and EtOH-SIL were found to be highly significant while compared to EtOH with the p-value of p< 0.0001.

Flow cytometric analysis of the effect of *Wolbachia* Surface Protein (WSP) on Mitochondrial Membrane Potential in HepG2 cells exposed to Ethanol

We examined the effect of *Wolbachia* Surface Protein (WSP) on Mitochondrial membrane potential in HepG2 cells by incubating with and without 56.57 mM Ethanol (EtOH) discretely for 24 h followed by treatment with 150 µg/ml *Wolbachia* Surface Protein (WSP) and 250 µM Silymarin (SIL) discretely for 24 h. Then the Mitochondrial Membrane Potential was analyzed by JC-1 staining and was quantified using FACSCalibur flow cytometer (Becton Dickinson (BD) Biosciences, San Jose, CA, USA).

Cells were acquired for analysis by CellQuest Pro software (Becton Dickinson (BD) Biosciences, San Jose, CA, USA) and quantification of cells with mitochondrial membrane depolarization, as the percentage of the total cell population was performed. We analyzed the mitochondrial membrane depolarization using FACSCalibur flow cytometer by detection of red and green fluorescence.

We observed that the level of mitochondrial membrane depolarization was little in Untreated (UT) HepG2 cells. Mitochondrial membrane depolarization was observed significantly in cells exposed to Ethanol (EtOH) for 24 h. *Wolbachia* Surface Protein (WSP) treatment for 24 h reduced the Mitochondrial membrane depolarization in Ethanol exposed HepG2 cells and Silymarin (SIL) treatment for 24 h also reduced the Mitochondrial membrane depolarization in Ethanol exposed HepG2 cells as determined by flow cytometer using JC-1 stain (Figure 6, 7, 8, 9) (Table 2) (Figure 10).

We found that the treatment of Ethanol exposed HepG2 cells with the *Wolbachia* Surface Protein (WSP) decreased EtOH-induced mitochondrial membrane depolarization. Therefore, in summary, the present study suggests that *Wolbachia* Surface Protein (WSP) protected HepG2 cells against mitochondrial membrane depolarization induced by Ethanol. These data suggest that *Wolbachia* Surface Protein (WSP) might act as cytoprotective agent.

Literature study showed that *Wolbachia* Surface Protein (WSP) hinders apoptosis in human Polymorphonulear cells (PMNs) [2]. However, there was no experimental evidence regarding WSP's (of *Wolbachia* of Uzifly) protection against Ethanol induced Mitochondrial membrane depolarization in HepG2 cells with respect to flow cytometric determination. These findings clearly showed that WSP could protect the HepG2 cells from mitochondrial membrane depolarization and cytotoxic effects of Ethanol. WSP has a similar action as that of the known hepatoprotective agent, Silymarin [14]. This study suggests that *Wolbachia* Surface Protein (WSP) may be used as an effective therapeutic drug to treat Ethanol-related liver diseases (Table 2) (Figures 6-10).

CONCLUSION

The present study showed that WSP exhibits cytoprotective effect against Ethanol-induced Apoptosis and Mitochondrial membrane depolarization in HepG2 cells by decreasing the Apoptosis and depolarization of mitochondrial membrane. Thus, the present study suggests that *Wolbachia* Surface Protein (WSP) may be used as a therapeutic drug to treat Ethanol-related liver diseases.

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